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(54) Title: BIODEGRADABLE AND BIOCOMPATIBLE POLYMERIC MICROSPHERES ENCAPSULATING <I>SALMONELLA ENTERITIDIS</I>BACTERIA

(57) Abstract: Biodegradable and biocompatible polymeric microspheres encapsulating *Salmonella enteritidis* are administered to chickens to provide the chickens with sustained protection against *S. enteritidis* infections.

BIODEGRADABLE AND BIOCOMPATIBLE POLYMERIC MICROSPHERES ENCAPSULATING *SALMONELLA ENTERITIDIS* BACTERIA

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to compositions and methods for providing chicken with sustained protection from infection with *Salmonella enteritidis*. More specifically, this invention relates to biodegradable and biocompatible polymeric microspheres encapsulating *S. enteritidis* which can be administered to chickens to protect them from *S. enteritidis* infections.

Background Art

Salmonella enteritidis, an agent which causes salmonellosis in poultry, can be transmitted vertically from laying hens to eggs. Consumption of eggs or meat contaminated with the organism can lead to food poisoning in humans. This is a worldwide problem in public health; in the U.S. alone, more than a million cases of salmonellosis are reported annually. Outbreaks in the elderly and in young children can be especially dangerous, resulting in severe gastroenteritis and possibly fatal septicemia.

In view of the large number of cases of *Salmonella enteritidis* cases reported each year, there is an obvious need for a reliable method for controlling the spread of *Salmonella* intestinal pathogens in poultry and for preventing the transmission of the pathogens into their eggs. Avirulent and killed injectable *S. enteritidis* vaccines have been developed and are useful. These vaccination processes, however, are known to cause a large amount of stress to the chicken as multiple vaccinations are required.

There thus is a significant need for a method by which poultry can be immunized against *S. enteritidis* in a simple, single dose, efficient, and cost-effective manner.

SUMMARY OF THE INVENTION

In accordance with the present invention, there are provided long term release, biocompatible and biodegradable, porous, polymeric microcapsules comprising whole killed *S. enteritidis* bacteria. Upon administration, the *S. enteritidis* is released in a biphasic pattern, wherein about 20% to about 50% of the *S. enteritidis* is released in an initial burst over the period of about 7 to 15 days, followed by the release of the remaining *S. enteritidis* in a steady and sustained manner over a period of about 70 to about 100 days. The microcapsules can be made by a (water-in-oil)-in-water emulsion process.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A and 1B show the size distribution and morphology of *S. enteritidis*-loaded microspheres formed using PLGA 65:35 and PLGA 50:50, respectively.

Figure 2 shows the size distribution and morphology of *S. enteritidis*-loaded microspheres formed using PLGA 50:50.

Figures 3A and 3B show the size distribution and morphology of *S. enteritidis*-loaded microspheres formed using PLGA 65:35 after release.

Figure 4 shows the *in vitro* release profile of *S. enteritidis*-loaded microspheres (calculated in 3 mg of the microspheres) formed using PLGA 65:35 after release.

Figure 5 shows antibody response in chicken treated with the *S. enteritidis*-loaded microspheres formed using PLGA 65:35.

DETAILED DESCRIPTION OF THE INVENTION

Microencapsulation is a process whereby very small droplets or particles of an active or otherwise desirable substance are distributed uniformly in a polymer matrix which is essentially inert and serves to isolate or protect the desirable substance. The desirable material is released from the polymeric matrix through erosion, permeation or rupture of the matrix. Variation in the size or material of the matrix and in the method by which the microcapsules are made can be utilized to control the rate or timing of the release of the core material. In the following

description, the compositions of the present invention are described as microspheres. As used herein, this term encompasses microcapsules and microparticles. The term also encompasses "sub micron" spheres, i.e., spheres between about 0.1 μm and 10 μm in diameter.

In accordance with the present invention, it now has been discovered that microspheres containing whole, killed *S. enteritidis* can be administered to poultry to protect the poultry from *S. enteritidis* infection. One dose of the vaccine of this invention has been found to provide poultry with protection from *S. enteritidis* infection as soon as 3-4 weeks post-administration and lasting for a period of at least six months.

The microspheres of the present invention are characterized as having a biphasic release pattern. Specifically, the microspheres provide a high initial release rate of about 20% to about 50% of the total *S. enteritidis* in the microspheres for a period of about 7 to about 15 days, preferably about 7-10 days followed by a lower, steady rate of release of the remaining *S. enteritidis* which occurs over a longer period of time, as described in detail below.

A preferred method for forming the microspheres of the present invention is to dissolve a polymer of choice in a solvent or solvents. More specifically, the preferred method uses a technique known as the double emulsion method (also called the complex emulsion method). In the double emulsion method, an aqueous solution of the *S. enteritidis* bacteria is emulsified with a larger quantity of a non-aqueous solution of a selected polymer. The emulsion can be

made by sonication or by homogenization. A preferred solvent is methylene chloride. Other suitable solvents include ethyl acetate, acetone, tetrahydrofuran and chloroform. The volume:volume ratio of aqueous solution to non-aqueous solution generally is within the range of 1:80 to about 1:12, and preferably is within the range of about 1:40 to about 1:12.

Choice and amount of solvent can affect the size and release profile of the resulting microspheres. The amount of CH_2Cl_2 or other solvent affects the viscosity of the polymer solution. Using a relatively low amount of CH_2Cl_2 will provide a more viscous polymer solution, ultimately resulting in bigger microspheres and, eventually, a longer overall time period of *S. enteritidis* release. Increasing the amount of CH_2Cl_2 relative to the amount of polymer used will result in the ultimate formation of more porous (and, hence, faster releasing) microspheres.

Desirably, the concentration of polymer in solution is within the range of about 12 to about 150 mg/ml and preferably is within the range of about 30 to about 80 mg/ml. These suggested concentrations can be modified, as suggested above, if it is desired to significantly change the *S. enteritidis* release profile. Also desirably, the aqueous solution of *S. enteritidis* is within the range of about 20 mg/ml to about 600 mg/ml and preferably is within the range of about 30 to about 400 mg/ml. The weight:weight ratio of polymer to *S. enteritidis* affects the release rate of the *S. enteritidis*. The release rate increases slightly as the ratio decreases. The ratio of the

range of polymer weight to *S. enteritidis* weight is within 1.5:1 to 40:1 and preferably is within the range of about 4:1 to about 40:1.

The resulting emulsion then is emulsified further by mixing (microspheres) or by either sonication or homogenization (sub-micron spheres) in a still larger quantity of an aqueous solution, forming a (water-in-oil)-in-water double emulsion. A preferred solution for forming the second emulsion is phosphate buffered saline (PBS), which further can comprise an emulsifier. Suitable emulsifiers include polyvinyl alcohol (PVA), span 80 and Tween 80. Suitable amounts of emulsifier generally are within the range of about 0.01% to about 5% by weight; a preferred amount of PVA is about 0.05% by weight for microspheres and about 0.5% by weight for sub-micron spheres. The higher the emulsifier concentration, the smaller the sphere size, which will result in a faster release profile of the *S. enteritidis*.

Changing the volume ratio of first emulsion to second emulsion can affect the removal rate of the solvent. The removal rate of the solvent is increased as this ratio decreases, resulting in a denser skin layer of microsphere and further in a slow release of *S. enteritidis*. The volume ratio of first emulsion to second emulsion is within the range of about 1:500 to about 1:1.5. Preferably the ratio is within the range of about 1:50 to about 1:1.5.

The polymer solvent then is allowed to be removed by evaporation and/or extraction, hardening the polymer and encapsulating the inner water droplets which contain the bacteria into small microspheres, generally

about 0.1 to about 400 µm median diameter, preferably about 5 to about 120 µm median diameter. When hardening is complete, the microspheres are filtered (microspheres) or centrifuged (sub-micron spheres), washed and dried. Filtering can be with a 0.02 µm to a 20 µm mesh, preferably a 5 µm mesh. Washing can be carried out using about 15 ml to about 5000 ml water, typically with about 100 ml to about 500 ml phosphate buffered saline (PBS). Drying can be accomplished using such methods as vacuum drying, lyophilization and fluidized bed drying in accordance with conventional techniques.

Desirably, the microspheres produced in accordance with this invention comprise about 25 µg to about 400 µg *S. enteritidis* per mg of spheres. Typically, the microspheres are administered such that the animal will receive a total dose of the killed *S. enteritidis* of at least about 0.15 mg. Preferably, a total dose of about 0.15 mg to about 15 mg is administered per chicken.

The release of the *S. enteritidis* can occur by two different mechanisms. The *S. enteritidis* can be released by diffusion through aqueous-filled channels in the polymeric matrix which are the result of voids or pores created by the internal aqueous phase or by solvent removal during the formation of the microspheres. The porosity of the microspheres result in a high initial burst of *S. enteritidis* release upon administration of the microspheres, as *S. enteritidis* at or near the surfaces of the pores of the microspheres dissolves in body fluids which enter the pores and is carried away from the spheres. In accordance with the present invention, approximately

20% to about 50% of the total encapsulated *S. enteritidis* is released in the initial burst, which typically takes place over a period of about 7 to about 15 days. By achieving such an initial release rate, poultry administered the microspheres show an obvious antibody response only 3-4 weeks post-injection. This is an advantage over immunization methods known in the art which have not resulted in a clear antibody response until 50 days post-immunization.

A second mechanism of release is the release of *S. enteritidis* due to degradation of the polymer. This follows the initial burst release of *S.* enteritidis, and results in the remaining *S. enteritidis* in the microspheres being released more gradually. Typically, the remaining *S. enteritidis* is released steadily over a period of at least about 10 to about 15 weeks. The rate of degradation can be controlled by changing polymer properties that influence the rate of hydration of the polymer. These properties include, for example, the choice of monomers used, the ratio of different monomers which comprise the polymer, and the molecular weight of the polymers. These properties can affect the hydrophilicity and crystallinity of the polymer, which in turn control the rate of hydration of the polymer. Hydrophilic excipients, such as carbohydrates, surfactants, polyethylene glycol (PEG) and low molecular weight polyesters also can be incorporated in the polymer to increase hydration and alter the rate of erosion of the polymer.

Polymers useful in the present invention include poly(lactide) (PLA), polyglycolide (PLG), poly(lactide-co-glycolide) copolymers (PLGA), polyethylene glycol

(PEG), polyorthoesters, polyanhydrides and polyphosphoesters. Preferred polymers are poly(lactide-co-glycolide) copolymers. Although the polymer can be all poly(lactide) or all polyglycolide, a blend of the two polymers (PLGA copolymers) preferably is used, and that copolymer preferably has a composition which is within the range of about 65:35 to about 50:50. Suitable molecular weights of the polymers are within the range of about 10,000 to about 110,000. Desirably, the molecular weight is about 40,000 to about 75,000 for 50:50 compositions and about 10,000 to about 75,000 for 65:35 compositions.

By varying the properties of the polymer, i.e., its composition, molecular weight, or its nature, one can affect the release rate, the proportion of bacteria released by diffusion vs. degradation, and the overall release period. For example, varying the molecular weight of the PLGA or the content of lactide in the polymer affects the rate at which the microspheres will degrade in body fluids. Increasing the glycolide content of a poly(lactide-co-glycolide) polymer and decreasing the molecular weight of the polymer can enhance the hydrolysis of the polymer and so provide increased *S. enteritidis* release from polymer erosion. Using higher molecular weights of PLGA, e.g., in the range of about 75,000 to about 110,000, or using a content of lactide to glycolide of at least about 75:25, the period of *S. enteritidis* release following the initial burst can be extended. However, a larger volume of internal aqueous solution desirably is used to achieve more porous microspheres which can still result in a desirable initial release even with PLGA

polymers comprising a high content of polylactide to polyglycolide. Also, the degradation rate of the microspheres can be adjusted by using a combination of two different kinds of polymers. For example, the degradation rate of polycaprolactone (PCL) is much slower than that of PLGA; the degradation rate of PEG is much faster. Microspheres fabricated with a blend of PEG and PLGA will have a much faster degradation rate and a faster rate of total release of *S. enteritidis*. PEG has little effect on the initial burst of release; the release of *S. enteritidis* in the initial burst is controlled by the porosity of the microspheres.

Polyorthoesters and polyanhydrides also can be used to deliver *S. enteritidis* in a biphasic release pattern as described above for PLGA. Persons of skill in the art can design the composition of the polymeric matrix to have a specific desired release profile given the guidance provided herein.

The pH of the formulations of this invention is generally in the range of about 5 to about 8.

The formulations of the present invention can comprise other components in addition to the *S. enteritidis*, provided that any such additional component does not interfere with the *S. enteritidis* or its release from the microspheres and is provided in an amount suitable for safe and effective pharmaceutical administration. Such additional components can include adjuvants, nutrients, drugs, peptides, and immunomodulators. Useful adjuvants include aluminum hydroxide, latex particles or liposomes, which adsorb

antigen and enhance immune responses. Other useful adjuvants can include saponin and mineral oil.

Peptides of interest include muramyl dipeptide (MDP), which enhances antibody production and stimulates and activates macrophages. Trehalose dimycolate also can be included to stimulate macrophages.

Immunomodulators which can be provided in the compositions of the present invention include cytokines, which have a complex activity to stimulate phagocytosis by macrophages or neutrophils, increase the activity of natural killer cells and increase production of other cytokines. Another useful immunomodulator is tumor necrosis factor (TNF). Certain complex carbohydrates, such as Zymogen, glucan, dextran sulfate and lectinans, also can be included to serve as immunostimulators and to activate macrophages.

To administer the microspheres of the present invention, a suitable amount of the microspheres can be suspended in a PBS solution and the resulting suspension is administered by injection or orally to poultry. A suitable dose comprises about 0.15 mg to about 15 mg of the killed, whole *S. enteritidis*. The injections are administered intramuscularly or subcutaneously. Oral administration can be by dropper or by mixing the suspension of microspheres in poultry feed.

As indicated above, it has been found that a single dose comprising an amount of *S. enteritidis* within the ranges set forth above is sufficient to provide at least about 6 months of protection from

infection by *S. enteritidis*. Protection is effective within about 3-4 weeks of immunization.

EXAMPLES

Example 1

Preparation of PLGA 65:35 Microspheres with 5% (wt) of Loading of *S. enteritidis*

Six hundred mg of polymer PLGA 65:35 were as dissolved in 12 ml of CH_2Cl_2. To this solution was added 0.5 ml of a *S. enteritidis* aqueous suspension (72 mg/ml, internal aqueous phase) to produce the primary emulsion by sonication. The resulting emulsion was injected with stirring into 250 ml of phosphate buffered saline (PBS) containing 0.05% wt of polyvinyl alcohol (PVA) as an emulsifier at 15°C to produce a double emulsion. Then 640 ml of PBS containing 0.05% wt of PVA was added to this solution for 4 hours in order to extract CH_2Cl_2 into the external phase. The resulting *S. enteritidis*-containing microspheres were filtered, washed with PBS, and vacuum-dried. The microspheres ranged in size from 0.5 to 120 μm, which were suitable for injection dosage. Figure 1A shows the size distribution and morphology of the microspheres. It is clear that *S. enteritidis*-containing microspheres produced had a porous structure. The porous structure will allow a high initial release of *S. enteritidis* once the microspheres are administered, which, in turn, will result in a high response of antibody.

Example 2

PLGA 65:35 Microspheres with 10% (wt) loading of *S. enteritidis*

600 mg of polymer PLGA 65:35 were dissolved in 12 ml of CH_2Cl_2. To this solution was added 0.5 ml of a suspension of *S. enteritidis* in water (144 mg/ml) to produce the primary emulsion by sonication. The other steps were the same as in Example 1, above. The resultant microspheres ranged in size from 0.5 to 120 µm, suitable for injection dosage.

Example 3

PLGA 50:50 Microspheres with 5% (wt) loading of *S. enteritidis*

600 mg of PLGA 50:50 were dissolved in 12 ml of CH_2Cl_2. To this solution was added 0.5 ml of a suspension of *S. enteritidis* in water (72 mg/ml) to produce the primary emulsion by sonication. The remaining steps were the same as in Example 1, above. The majority of the microspheres produced ranged in size from 40 to 80 µm, suitable for injection. The size distribution and morphology of the microspheres are demonstrated in figure 1B. As in Example 1, the microspheres had a porous morphology.

Example 4

PLGA 50:50 Microspheres with 10% (wt) or loading of *S. enteritidis*

600 mg of PLGA 50:50 were dissolved in 12 ml of CH_2Cl_2. To this solution was added 0.5 ml of a suspension of *S. enteritidis* in water (144 mg/ml) to

produce the primary emulsion by sonication. The remaining steps were the same as in Example 1, above. The majority of the microspheres had a size in the range of about 40 to about 80 µm, suitable for injection dosage.

Example 5

PLGA 50:50 Submicrospheres with 10% (wt) loading of *S. enteritidis*

400 mg of PLGA 50:50 were dissolved in 4 ml of CH_2Cl_2. To this solution was added 0.1 ml of a suspension of *S. enteritidis* in water (400 mg/ml) to produce the primary emulsion by sonication. The emulsion was mixed with 6 ml of PBS containing 0.5% PVA to produce a double emulsion by sonication. The double emulsion was poured into 150 ml of PBS containing 0.05% PVA with stirring. The 250 ml of PBS containing 0.05% wt PVA were added to the solution for 2 hours in order to extract CH_2Cl_2 into the external phase. The resulting *S. enteritidis*-containing microspheres were centrifuged, washed with PBS and vacuum dried. The resulting microspheres ranged in size from about 0.1 to about 10 µm, which are easily taken up by Peyer's patches in the intestine and are suitable for oral administration. The distribution and morphology of the microspheres is shown in Figure 2.

Example 6

In Vitro Release Kinetics

30 mg of the microspheres produced in Example 1 were suspended in 1 ml of PBS pH 7.4 at 37° C. The supernatant was removed using a syringe and replaced with fresh PBS at regular intervals. The content of antigen in the supernatant was measured using an ELISA. The results showed that the duration of *S. enteritidis* release by the microspheres was more than two months and there was a high initial release of 110 µg/5 mg microspheres over 10 days. This initial release was followed by more than three months of steady release of the remaining *S. enteritidis*. Figure 3 shows the change in size distribution and morphology after release. Figure 4 shows the *in vitro* release profile (calculated in 3 mg of the microspheres) of the specific microspheres composed of PLGA 65:35 produced in Example 1.

Example 7

Chicken Test 1

5 mg of the *S. enteritidis*-loaded microspheres produced in Example 1 were suspended in 150 µl of PBS, then injected into 6 two-day old chicks. Blood samples of the treated chicks were taken at one or four weeks post-injection. Figure 5 shows the antibody response of the chicks post-treatment with the *S. enteritidis*-loaded microspheres. The microspheres successfully induced an antibody response in the chicks within four weeks which was maintained for six months.

Example 8
Chicken test 2

The following formulations were prepared and administered:

1. 5 mg. of *S. enteritidis*-loaded microspheres made in accordance with Example 2 were suspended in 150 µl of distilled water and injected intramuscularly in each of six 7-day old chicks.

2. 5 mg. of *S. enteritidis*-loaded microspheres made in accordance with Example 4 were suspended in 150 µl of distilled water and injected intramuscularly in each of six 14-day old chicks.

3. 9 mg of *S. enteritidis*-loaded microspheres made in accordance with Example 5 was administered orally by dropper to six 14-day old chicks.

4. A fourth group of six 14-day old chicks was used as a control group and did not receive any *S. enteritidis*-loaded microspheres.

Seven day old chicks were used in (1) above to investigate whether chick age had any effect on immunization.

A month after the *S. enteritidis* administration, the chicks in each of the three treatment groups, as well as the control group, were challenged with 5×10^7 live *S. enteritidis*. All of the chicks in group 4 became sick and showed clinical signs such as manifest somnolescence, weakness, loss of appetite and adherence of chalky white material to the vent. All of the

chicks treated with the *S. enteritidis* polymeric microspheres, however, showed no symptoms of disease. The results of the challenge are presented in Table 1 below. From these results, it is clear that the *S. enteritidis* microspheres of the present invention protect chicks from live *S. enteritidis* challenge.

Table 1

Challenge Results of Chickens Following Treatment with the *S. enteritidis* Microspheres

Experimental Group	Chicken Number	Age (days)	Sick
Group 1	6	7	0/6
Group 2	6	14	0/6
Group 3	6	14	0/6
Group 4	6	14	6/6

Claims:

1. A composition comprising polymeric microspheres encapsulating *S. enteritidis*, wherein the *S. enteritidis* is released from the microspheres in a biphasic pattern characterized by a first phase wherein about 20% to about 50% of the total *S. enteritidis* is released in an initial burst over a period of about 7 to about 15 days and a second phase, which follows said first phase, wherein the remainder of the *S. enteritidis* is released over a period of about 10 to about 15 weeks.

2. The composition of claim 1, wherein the microspheres comprise poly(lactide) (PLA), polyglycolide (PLG), poly(lactide-co-glycolide) copolymers (PLGA), polyorthoesters, polyanhydrides, polyphosphoesters, or a combination thereof.

3. The composition of claim 2, wherein the microspheres comprise poly(lactide-co-glycolide) copolymers.

4. The composition of claim 3, wherein the ratio of lactide to glycolide ranges from about 80:20 to about 50:50.

5. The composition of claim 4, wherein the ratio of lactide to glycolide ranges from about 65:35 to about 50:50.

6. The composition of claim 3, wherein the molecular weight of the poly(lactide-co-glycolide) ranges from about 10,000 to about 110,000.

7. The composition of claim 1, wherein the microspheres have a diameter of about 0.1 μm to about 400 μm.

8. The composition of claim 7, wherein the microspheres have a diameter of about 5 μm to about 120 μm.

9. The composition of claim 1, wherein the microspheres further comprise at least one adjuvant, nutrient, drug, peptide, immunomodulator, or combination thereof.

10. The composition of claim 1, wherein the amount of *S. enteritidis* incorporated into the polymer is about 25 μg to about 400 μg per mg of polymer.

11. A method of making polymeric microspheres comprising *S. enteritidis* distributed throughout, which comprises

(a) emulsifying an aqueous solution of *S. enteritidis* with a non-aqueous solution of polymer; said non-aqueous solution having a weight:volume ratio of about 12 mg/ml to about 150 mg/ml; wherein the weight:weight ratio of the *S. enteritidis* to polymer is about 1:40 to about 1:1.5 and the volume:volume ratio of aqueous medium to non-aqueous medium is about 1:80 to about 1:12;

(b) mixing the resultant water-in-oil emulsion from step (a) with a second aqueous solution so as to form a (water-in-oil)-in-water double emulsion;

(c) removing the polymer solvent to produce porous microspheres; and

(d) filtering or centrifuging, washing and drying the microspheres.

12. The method of claim 11, wherein the polymer comprises poly(lactide) (PLA), polyglycolide (PLG), poly(lactide-co-glycolide) copolymers (PLGA), polyethylene glycol (PEG), polyorthoesters,

polyanhydrides, polyphosphoesters, or a combination thereof.

13. The method of claim 11, wherein the polymer solvent comprises CH_2Cl_2, ethyl acetate, acetone, tetrahydrofuran or chloroform.

14. The method of claim 11, wherein the second aqueous solution comprises phosphate buffered saline or distilled water.

15. The method of claim 14, wherein the second aqueous solution further comprises an emulsifier.

16. The method of claim 15, wherein the emulsifier comprises polyvinyl alcohol, span 80 or Tween 80.

17. The method of claim 11, wherein the microspheres have a median diameter of about 0.1 μm to about 400 μm.

18. The method of claim 11, wherein the microspheres comprise poly(lactide-co-glycolide) copolymers.

19. The method of claim 18, wherein the ratio of lactide to glycolide ranges from about 80:20 to about 50:50.

20. The method of claim 19, wherein the ratio of lactide to glycolide ranges from about 65:35 to about 50:50.

21. The method of claim 18, wherein the molecular weight of the poly(lactide-co-glycolide) copolymers ranges from about 10,000 to about 110,000.

22. The method of claim 11, wherein the microspheres further comprise at least one adjuvant, nutrient, drug, peptide, immunomodulator, or combination thereof.

23. The method of claim 11, wherein the amount of *S. enteritidis* incorporated into the polymer is about 25 µg to about 400 µg per mg of polymer.

24. A method of immunizing poultry from infection from *S. enteritidis* which comprises administering to poultry an effective amount of the microspheres of claim 1.

25. The method of claim 24, wherein the microspheres are administered in a physiologically acceptable suspension.

26. The method of claim 25, wherein the suspension is administered by injection or orally.

27. The method of claim 24, wherein the poultry each receive a single dose of about 0.15 mg to about 5 mg.

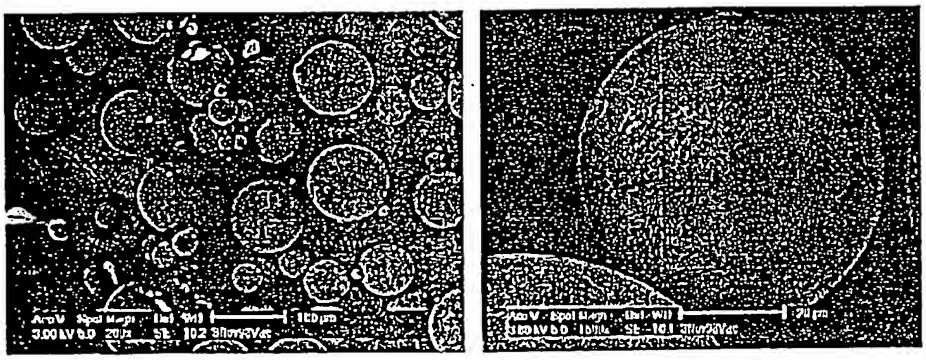
28. The method of claim 24, wherein the poultry exhibit an effective *S. enteritidis* antibody response 3-4 weeks post-microsphere administration which is maintained for at least about 6 months.

29. A method for immunizing poultry from infection from *S. enteritidis* which comprises administering to poultry a single dose of a suspension of microspheres comprising killed *S. enteritidis* encapsulated in a porous polymeric shell comprising poly(lactide-coglycolide) copolymers; said microspheres comprising a total dose of *S. enteritidis* of about 0.15 mg to about 15 mg; wherein the *S. enteritidis* is released from the microspheres in a biphasic pattern upon administration wherein about 20% to about 50% of the encapsulated *S. enteritidis* is released in a first phase in an initial burst within the first 7 to about

15 days of the administration of the suspension and the remainder of the *S. enteritidis* is released in a second phase over a period of about 10 to about 15 weeks.

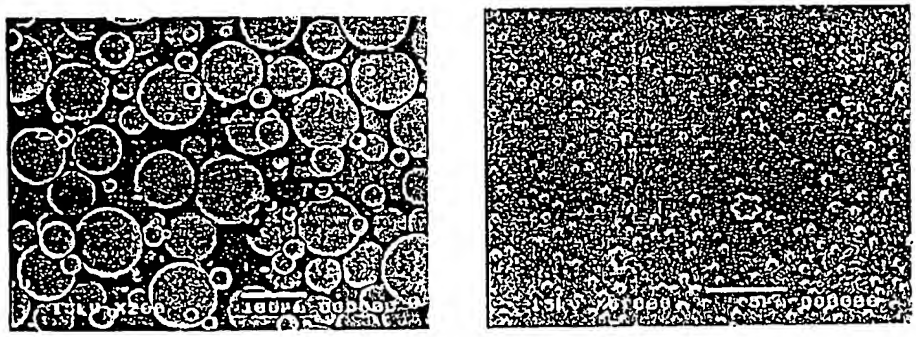
30. The method of claim 29, wherein the immunization is effective within about 3-4 weeks of the administration of the microspheres.

Figure 1



PLGA 65:35, 200 X, scale bar: 100 μm PLGA 65:35, 1500 X, scale bar: 20 μm

A

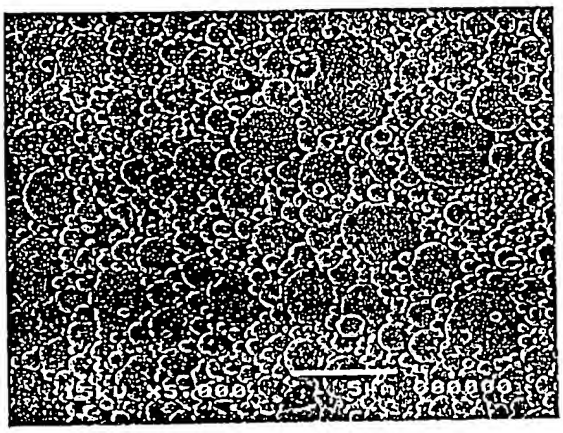


PLGA 50:50, 200 X, scale bar: 100 μm PLGA 50:50, 5000 X, scale bar: 5 μm

B

Fig. 1 SEM micrographs of *Salmonella* enteritidis-loaded microspheres composed of PLGA 65:35 (A), PLGA 50:50 (B)

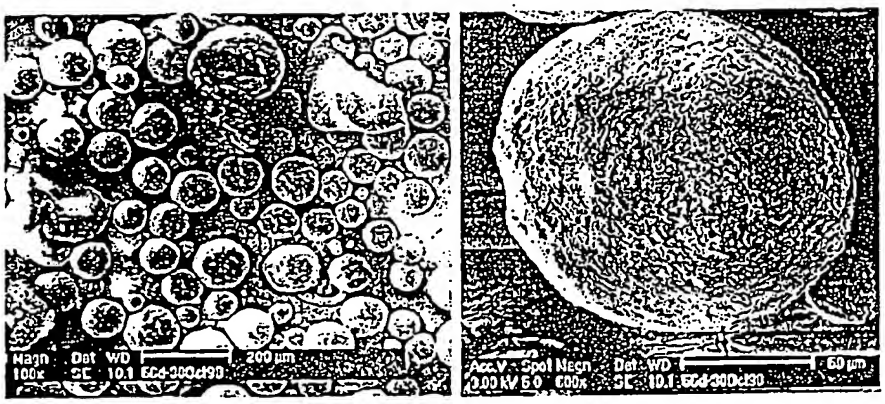
Figure 2



PLGA 50:50, 5000 X, scale bar: 5 μm

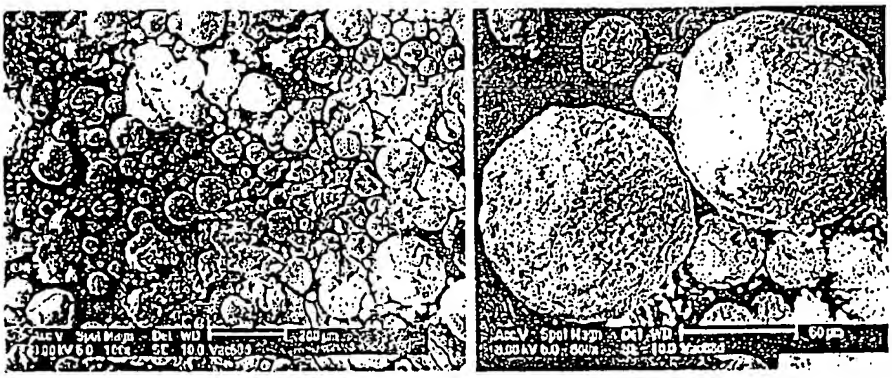
Fig. 2 SEM micrographs of *Salmonella* enteritidis-loaded microspheres/nanospheres composed of PLGA 50:50.

Figure 3



100 X, scale bar: 200 μm 600 X, scale bar: 50 μm

A: PLGA 65:35, released for 56 days



100 X, scale bar: 200 μm 500 X, scale bar: 50 μm

B: PLGA 65:35, released for 68 days

Fig. 3 SEM micrographs of *Salmonella* enteritidis –loaded microspheres composed of PLGA 65:35 after release. A: 56 days, B: 68 days

Figure 4

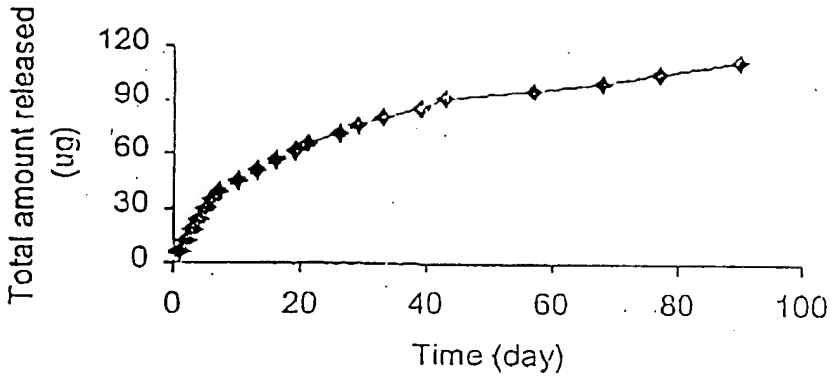


Fig. 4 *In vitro* release profile of the specific *Salmonella*-containing PLGA 65:35 microspheres (calculated in 3 mg of the microspheres)

Figure 5

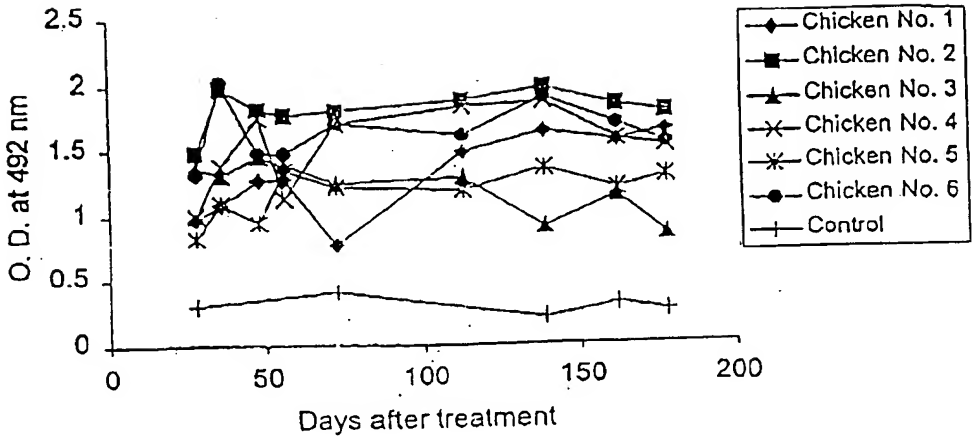


Fig. 5 Antibody response (O.D. Reading) in chickens treated with the specific *Salmonella* PLGA 65:35 microspheres.

INTERNATIONAL SEARCH REPORT

Intl. Ional Application No
PCT/SG 00/00017

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K35/74 A61K39/112 A61K9/16

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HAZRATI, A.M. ET AL.: "Salmonella enteritidis vaccine utilizing biodegradable microspheres" PROCEEDINGS OF THE CONTROLLED RELEASE SOCIETY , vol. 20, 1993, pages 101-102, XP000952506	1-6,10, 24-30
A	abstract	11-23

X	EP 0 300 102 A (STOLLE RES & DEV) 25 January 1989 (1989-01-25)	1,2,7
A	page 2, line 42 - line 53 page 3, line 10 - line 56 page 4, line 24 - line 29 page 5, line 40 - line 46 page 6, line 16 - line 35 page 7, line 2 - line 21 page 7, line 39 - last line; claims 1-7,9,10,13; example 2; table I	3-6,8-30

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 October 2000	20/10/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Marttin, E

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INTERNATIONAL SEARCH REPORT

Int. tional Application No
PCT/SG 00/00017

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99 43349 A (DEGLING WIKINGSSON LENA ;SJOEHOLM INGVAR (SE)) 2 September 1999 (1999-09-02) page 4, line 4 - line 15 page 4, line 26 -page 5, line 3 page 5, line 27 - line 30; claims; example 2 ---	1-10,29, 30
A	YAN C ET AL: "CHARACTERIZATION AND MORPHOLOGICAL ANALYSIS OF PROTEIN-LOADED POLY (LACTIDE-CO-GLYCOLIDE) MICROPARTICLES PREPARED BY WATER-IN-OIL-IN- WATER EMULSION TECHNIQUE" JOURNAL OF CONTROLLED RELEASE,NL,ELSEVIER SCIENCE PUBLISHERS B.V. AMSTERDAM, vol. 32, no. 3, 1994, pages 231-241, XP002914206 ISSN: 0168-3659 abstract page 231, column 1, paragraph 1 -column 2, last paragraph page 232, column 1, line 9 - line 30 page 232, column 2, last paragraph -page 233, column 1, paragraph 1 page 240, column 1, last paragraph -column 2, paragraph 1 -----	11-23

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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.2

Present claims 1-10, 29 and 30 relate to a product defined by reference to a desirable characteristic or property, namely microspheres releasing Salmonella enteritidis in a biphasic pattern, with a first phase wherein 20-50% of the total S. enteritidis is released in 7 to about 15 days and a second phase wherein the remainder is released over a period of about 10 to 15 weeks. Present claim 30 relates to a product further defined by reference to a desirable characteristic or property, namely a method for immunizing poultry wherein the immunization is effective within 3-4 weeks of the administration of the microspheres. The product is defined by the release pattern of Salmonella enteritidis and the time to obtain effective immunization in poultry. However, a product cannot be sufficiently characterised by its release pattern or by the time to obtain immunization, because it is impossible to know which products have such a release pattern.
The claims cover all products having this characteristic or property, whereas the application provides support within the meaning of Article 6 PCT and/or disclosure within the meaning of Article 5 PCT for only a very limited number of such products. In the present case, the claims so lack support, and the application so lacks disclosure, that a meaningful search over the whole of the claimed scope is impossible. Independent of the above reasoning, the claims also lack clarity (Article 6 PCT). An attempt is made to define the product by reference to a result to be achieved. Again, this lack of clarity in the present case is such as to render a meaningful search over the whole of the claimed scope impossible. Consequently, the search has been carried out for those parts of the claims which appear to be clear, supported and disclosed, namely those parts relating to the products prepared in examples 1-5 and mentioned in claims 11-28, and the concept of "polymeric microspheres with a biphasic release of S. enteritidis".

The applicant's attention is drawn to the fact that claims, or parts of claims, relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inta ional Application No
PCT/SG 00/00017

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0300102 A	25-01-1989	AT 87215 T	15-04-1993
		AU 605159 B	10-01-1991
		AU 7608687 A	27-01-1989
		DE 3785033 D	29-04-1993
		DE 3785033 T	07-10-1993
		HK 112493 A	29-10-1993
WO 9943349 A	02-09-1999	AU 2754599 A	15-09-1999